University of Nevada, Reno

Sensitivity and specificity of an in-clinic point-of-care qPCR test for the diagnosis of canine parvovirus

A thesis submitted in partial fulfillment
of the requirements for the degree of

Bachelor of Science in Veterinary Science and the Honors Program

by

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May, 2020

UNIVERSITY OF NEVADA RENO

THE HONORS PROGRAM

We recommend that the thesis
prepared under our supervision by

BREANNA K. KJOLL

entitled

Sensitivity and specificity of an in-clinic point-of-care qPCR test for the diagnosis of canine parvovirus

be accepted in partial fulfillment of the
requirements for the degree of

BACHELOR OF SCIENCE, VETERINARY SCIENCE

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Michael B. Teglas, Ph.D., DVM, Thesis Advisor

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May, 2020

Abstract

Canine parvovirus (CPV) is a common disease found in dogs worldwide. Because of the widespread nature of CPV, quick and accurate detection of the pathogen is important. A variety of existing diagnostic tests utilize an antibody-based system that often trades accuracy for speed. A definitive diagnosis therefore requires direct detection through reference quantitative PCR (qPCR). However, qPCR is only available through specialized laboratories. This project aimed to compare the sensitivity and specificity of an in-clinic qPCR test to those of reference qPCR in diagnosing CPV. Fifty canine fecal samples were analyzed for CPV using an in-clinic test. Results were compared to those of a reference qPCR test. Sensitivity was 60.0% (CI 95%, 35.8%-80.2%), while specificity was 88.6% (CI 95%, 74.1%-95.5%) for all samples. The in-clinic test was easy to perform and had high specificity. However, the in-clinic test results still required further diagnostics for confirmation due to low sensitivity.

Acknowledgements

I would like to thank Credo Biomedical for funding and supporting this study by providing the in-clinic test and its associated materials and reagents. I would also like to thank the office of Undergraduate Research for providing funding through the Honors Undergraduate Research Award. Thanks also to VCA Baring Boulevard Animal Hospital, Animal Emergency and Specialty Center, Nevada Humane Society, and SPCA of Northern Nevada for their time and collection of the samples used in this study. Special thanks to my thesis advisor, Mike Teglas, for the opportunity to work on this project, and his guidance through every stage of the process, especially with campus closures. Last but not least, thank you to my parents for their unending encouragement and support.

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Introduction and Literature Review

Canine parvovirus (CPV) has been a common and important cause of illness and death in unvaccinated young dogs since it first emerged in the mid-1970s. It is a highly infectious and widely distributed disease, and one of the most common reasons for severe gastroenteritis in young dogs worldwide (Decaro et al., 2013). The canine parvovirus is extremely contagious and can reach up to 100% morbidity in unvaccinated populations (Nandi & Kumar, 2010). This is especially significant for animal shelters and similar settings where multiple dogs live in close proximity, since it makes outbreaks of CPV difficult to isolate and control.

Although CPV vaccines are very effective at preventing the disease, they are not required by the state of Nevada. This means that the unvaccinated population of the over 30,000 companion dogs in Nevada are at risk of CPV infection ("Common Diseases in Dogs & Cats in Nevada," 2019). Without proper, timely medical treatment, CPV infection can result in a mortality rate of 10% in adult dogs and up to 91% in puppies (Nandi & Kumar, 2010). Because of this, it's critical that sick animals are able to be isolated from other dogs and begin treatment as soon as possible to prevent further spread or death. The high mortality and morbidity rates associated with CPV mean that it is very important for veterinary hospitals to have easy-to-use, trustworthy tests for canine parvovirus.

High mortality and morbidity rates also mean that tests which often present false negatives, referred to as having low sensitivity, can present serious animal health issues by allowing a sick animal to continue to spread disease as well as preventing that animal

from receiving treatment. Although the consequences of a false negative test result seem obvious, a test which often gives false positive results, known as having low specificity, also presents problems. Treatment for CPV infection is intensive, meaning that unnecessary treatment will result in dog owners spending a large amount of money on treating their animal for a disease which the animal does not have. It also means that whatever disease is causing the dog to present at the hospital with CPV-like symptoms is continuing to go untreated. For these reasons, new testing methods for canine parvovirus must be tested extensively in order to ensure that the new test is able to accurately identify instances of CPV infection, with either lower or comparable rates of false positives and negatives.

This thesis focuses on a comparison of a new in-clinic test to the lab technique which serves as the diagnostic reference standard for confirming CPV infection. In evaluating the new in-clinic platform, it is valuable to understand other in-lab techniques that serve as diagnostic standards, as well as current in-clinic testing methods. Characteristics of canine parvovirus, including its evolutionary characteristics and subsequent variants, are also important factors.

Canine Parvovirus

Canine parvovirus is a single-stranded DNA virus that has a very high rate of genomic substitution, especially compared to related viruses like the feline panleukopenia virus (Decaro et al., 2009). According to Decaro et al. (2005b), the original CPV-2 virus emerged in the mid-1970s, with variants CPV-2a and CPV-2b virtually replacing the original strain shortly afterward. CPV types 2a and 2b have been found to have two

single nucleotide polymorphisms in the capsid protein gene sequence VP2. Although CPV-2a and -2b are still considered the most common parvovirus strains, CPV-2c is more virulent with a higher morbidity (Goddard & Leisewitz, 2010). The CPV-2c variant emerged in Italy in 2000 and has been shown to be more severe than either of the other two variants, with higher mortality rates as well as increased ability to infect vaccinated adult dogs (Decaro et al., 2009). Decaro et al. (2007) found that CPV-2c was spreading rapidly and had nearly replaced all incidence of CPV-2b in Italy seven years after the emergence of CPV-2c. It was also found to be spreading rapidly through the rest of Europe. In order to determine this spread, Decaro et al. (2005b) developed a qPCR assay to differentiate the three variants of CPV-2 with better accuracy. This made identifying the three strains for future studies much less difficult and allayed the concern that certain in-clinic tests were less able to detect CPV-2c (Decaro et al., 2013).

Canine parvovirus is spread through the fecal-oral route or via fomites to other susceptible dogs, helped by the stability of the virus in the environment (Decaro & Buonavoglia, 2017). Goddard & Leisewitz (2010) summarize the pathogenic and clinical characteristics of canine parvovirus. The virus is most common in puppies between the ages of six weeks and six months old and results in depression, lethargy, fever, vomiting, diarrhea, and leukopenia. Untreated, CPV can cause death in young dogs, making rapid response critical. Three to four days after infection, the virus can be found in the intestinal epithelium. The virus requires a host cell for replication, and this happens most effectively in rapidly dividing cells such as intestinal crypt epithelial cells. Viral particles are shed in the intestines and can be detected in the feces (Goddard & Leisewitz, 2010). Parvovirus spreads rapidly through populations of young dogs who do not have

immunity. Once acquired, clinical signs of CPV can be relatively nonspecific, including lethargy, vomiting, and diarrhea (Goddard & Leisewitz, 2010). Although these clinical signs, age of the animal, and vaccination history (or lack thereof) are useful for making a tentative diagnosis, more definitive diagnostic tests are necessary for detecting infection in a canine patient.

Occurrence of this disease is rare in canines that have received a full set of vaccinations. However, vaccines for CPV have been shown to interfere with diagnostic testing (Decaro & Buonavoglia, 2017; Freisl et al., 2017). In adult dogs which were vaccinated with a modified-live virus vaccine, nearly a quarter were found to be shedding CPV through their feces in the first month after vaccination (Freisl et al., 2017). Although viral concentration from recently vaccinated animals is generally lower than concentrations found in infected animals, this can lead to false positive results in both fecal antigen tests and viral DNA amplification tests (Decaro & Buonavoglia, 2017).

In-Clinic Testing

In veterinary clinics, enzyme-linked immunosorbent assay (ELISA) tests are often used to detect antibodies associated with the parvovirus pathogen (Waner, 2016). ELISA tests, such as the commonly used IDEXX SNAP test series, detect and measure the presence of parvovirus antigen in a fecal sample by binding the antigen to conjugate antibodies and assessing the binding activity using an enzyme ("Overview of ELISA – US," n.d.; SNAP Tests Use ELISA Technology—IDEXX US, n.d.). However, these tests can present false negatives when viral load is too small, before or after peak shedding of the virus. Due to the fact that modified-live vaccines against CPV also cause shedding of

parvovirus in the feces, samples from recently vaccinated animals may also test positive using these assays, causing false positives. Other tests commonly used to determine the presence of a parvovirus infection in veterinary clinics include immunochromatographic (IC) and hemagglutination (HA) tests. Immunochromatographic tests for parvovirus generally rely on the presence of antigen and run the analyte across a sheet that includes reactive molecules, conjugate proteins, and labels, which display a positive or negative result ("What is a lateral flow immunoassay and how does it work?," n.d.). Hemagglutination tests rely on the ability of viruses to attach to the surface of red-blood cells and cause blood cells to stick together, or agglutinate. However, all of these tests tend to have low sensitivity (Desario et al., 2005). Quantitative polymerase chain reaction, often referred to as qPCR, has been shown to be more sensitive than these other techniques for detecting viral presence in feces and is therefore considered the reference standard of detection (Goddard & Leisewitz, 2010).

Most current in-clinic point-of-care testing methods are ELISA tests such as the IDEXX SNAP or immunochromatography tests like the Zoetis Witness. As previously mentioned, both of these test types rely on the presence of parvovirus antigen in a sample, which then undergo a reaction with a conjugate and bind ("Frequently asked questions about SNAP Parvo Test," n.d.; "WITNESS Parvo," n.d.). In these in-clinic tests, a color change or the appearance of a line or spot occurs to indicate a positive result. According to one study by Desario et al. (2005), testing the Parvovirus IDEXX SNAP test, the ICPOC SNAP test was the most common, but least sensitive test due to the large amounts of viral antigen needed to be present in order for a sample to test positive. This study also evaluated hemagglutination (HA) and qPCR as testing methods

for parvovirus. Similar to qPCR, HA was only available in the laboratory. However, fresh blood was needed. Hemagglutination was also found to take more time and be less sensitive due to antibodies present in the gut lumen. Desario et al. (2005) overwhelmingly found that qPCR was the most sensitive test, establishing qPCR as the reference standard, based on Decaro et al.'s (2005) qPCR methodology. Another study by Decaro et al. (2013) examined the ability of an immunochromatographic ICPOC test, the Zoetis Witness test, to determine the presence of infection when different variants of the virus were present. The study found that, although the same sensitivity issues were present in the immunochromatographic ICPOC test, there was no significant difference in detection of CPV-2c compared to CPV-2a or -2b. Of these ICPOC tests, the ELISA and immunochromatography tests are used most frequently due to their speed and ease of use. However, they lack sensitivity, making qPCR more useful for definitive diagnosis.

Quantitative PCR

Quantitative PCR (qPCR) is a method used to amplify a particular sequence of DNA or RNA by rapidly copying a specific region until the sequence can be adequately detected through the use of fluorescent dyes or probes (Canene-Adams, 2013). Primers, which complement the gene of interest, allow a polymerase to bind and copy the gene. In qPCR that uses a SYBR green system, a dye reacts to the presence of double-stranded DNA when a gene is copied and fluoresces. When a fluorescent probe is used as the detection device, it binds downstream from the primer and fluoresces when the polymerase reaction has ended. Due to the fact that probes only fluoresce when there is genuine amplification of a target sequence, rather than simply in the presence of double-

stranded DNA, probes are generally preferred ("Real time PCR basic principles," n.d.). In a diagnostic context, this copying and detection process is used to detect a genetic region specific to that of the infectious agent, such as a viral or bacterial sequence. Because it detects the presence of the pathogen genomic material, qPCR testing does not rely on detecting antibodies created during an immune response. This increases the reliability of the test, since an immune response may take time to develop after initial infection and may also be present due to the body's response to a vaccine rather than infection by a live virus.

Feces is used in qPCR testing for CPV, due to shedding of the virus in the fecal material of infected animals (Decaro et al., 2013; Goddard & Leisewitz, 2010). Decaro et al. (2005a) developed a method of quantitative detection using a qPCR assay, which allowed for the best sensitivity and rapidity compared to other precise in-lab methods. In order to do this, the group used qPCR technology, utilizing a dual-labeled DNA probe rather than a less precise fluorescent dye like SYBR Green. They were able to develop specific primers for CPV DNA detection based on the conserved region on the VP2 gene, which is one of two genes that encodes the parvoviral capsid (Decaro et al., 2005a). Utilization of this technology allows for real-time optical monitoring of the qPCR reaction based on the release of fluorescing dyes and probes when viral DNA replication is detected. It also created a reference standard for the detection of CPV and established effective primers for qPCR of CPV DNA.

Although qPCR is the standard for diagnosis, it typically requires that samples be sent to specialized laboratories that have the capabilities to run this diagnostic test. This transport leads to delays before results can be made available. Delays may also result in

loss or destruction of DNA in the sample (Selder et al., 2017). Laboratory qPCR tests also require multiple steps, expensive equipment, and trained personnel. Although qPCR is considered the standard for diagnosis of canine parvovirus, many benefits have been shown to arise from the use of point-of-care testing.

In-Clinic Point-of-Care PCR

In-clinic point-of-care (ICPOC) testing can offer many benefits to reference laboratory testing. By producing results more quickly, treatment can be started earlier (Boyd and Woolley, 2016). Sensitive and specific diagnostic tests that can be performed easily in veterinary clinics would assist in the accurate diagnosis and subsequent treatment of veterinary diseases. This is especially important in diseases like canine parvovirus, in which starting treatment, such as fluid and electrolyte replacement, earlier can greatly improve the outcome for an infected dog. There is also an increasing need for low-cost reliable tests that are able to be performed in-clinic. This would make veterinary care more affordable and more feasible for remote areas, which may not be near a lab that can perform qPCR (Busin et al., 2016). However, as previously mentioned, many current in-clinic, point-of-care tests lack the sensitivity provided by a PCR-based method. Recently, more rapid point-of-care PCR tests have been developed and become available to clinics (Waner, 2016).

In order to simultaneously utilize the increased accuracy and precision of qPCR technology and the better efficiency and convenience of ICPOC tests, in-clinic qPCR tests have been developed over the last decade. These tests are intended to perform the thermal cycling, nucleic acid replication, and fluorescent detection of qPCR on a smaller

scale and in a less labor-intensive way, which would allow them to be conducted by veterinary staff. According to a review by Waner (2016), antibody tests such as ELISA and immunochromatographic tests have dominated in-clinic testing due to their rapidity and low cost. However, PCR testing has the ability to detect the virus before there is an antibody response. Waner argues that the rise of ICPOC PCR platforms will allow veterinary staff to make more accurate and quick diagnoses. In an earlier study by Waner et al. (2014), artificially infected canines were tested using both reference qPCR and a specific ICPOC PCR platform. Tests were performed during each stage of infection. The ICPOC PCR platform was found to have 100% specificity, meaning it correctly identified all of the truly negative samples. However, the same platform exhibited very low sensitivity (30%) except during the acute stage of the disease (75%), meaning it incorrectly labeled some truly positive samples as negative. This result was similar to those of Selder et al. (2018) in which another ICPOC PCR platform was compared to the reference standard. Samples were from naturally infected dogs and collected from area clinics. This study also evaluated the ease of performance and included information on true and false positives and negatives, as well as the negative and positive predictive values (NPV and PPV, respectively). The group found that the ICPOC PCR test was very easy to use and was able to release results in less than 75 minutes. Again, the specificity of the ICPOC PCR platform was 100%. However, the sensitivity of that ICPOC PCR platform was below 60% for each of the types of samples that were collected by the group. This finding shows that, despite the increased ease-of-use, ICPOC PCR platforms have not shown similarly high sensitivity to the reference qPCR.

Although more ICPOC PCR platforms have become available in the last five years, there is still very little research available on the use and efficacy of these ICPOC qPCR tests. Most research on in-clinic tests remains focused on newer or improved ELISA and immunochromatographic tests. However, it is important to continue testing a variety of in-clinic point-of-care qPCR tests in order to determine if these tests bring a higher level of accuracy direct to hospitals, based on the high level of accuracy associated with traditional qPCR. It is also important to contribute to the understanding of the practicality of use of ICPOC qPCR tests by evaluating their ability to detect diseases commonly seen in veterinary hospitals. This would determine whether these tests are a valuable investment for veterinary hospitals, which rely on getting timely and accurate results from in-clinic tests in order to pursue the correct treatment methods. Researching the efficacy of more ICPOC qPCR tests will also help medical equipment companies interested in developing these tests to better understand the value of their tests and how to improve them.

Methodology

This study investigated the sensitivity and specificity of an ICPOC qPCR test (the QubeMDx) in comparison with the reference standard of qPCR. The aim of this project was to understand the practicability of a point-of-care test like the QubeMDx in terms of the ease-of-use and accuracy of this method. It also examined the retest reliability when samples were retested using the QubeMDx. These comparisons were determined to be important to the evaluation of the overall efficacy of the test, especially in an in-clinic diagnostic context wherein tests must be convenient as well as accurate.

Based on similar comparisons of pathogen detection in ICPOC qPCR tests to the qPCR standard, it is predicted that the QubeMDx will have similar specificity to qPCR but will have lower sensitivity as found in other test systems (Selder et al., 2018; Waner et al., 2014). It is also expected that the QubeMDx will be significantly easier to use than the qPCR lab technique due to the pre-packaged test elements and shorter testing times. This would also be consistent with similar studies of ICPOC qPCR platforms, which found the in-clinic platform to be more user-friendly than the qPCR diagnostic standard (Selder et al., 2018).

In order to answer the questions of how the QubeMDx and the qPCR diagnostic standard compare, fecal samples were collected from within the northern Nevada region and tested by both the QubeMDx and traditional qPCR. This was done in order to determine the associations between positive and negative results in both machines as well as association between results in multiple trials of the QubeMDx. These methods were chosen based on precedent from similar studies, as statistical comparisons between the results of new tests and the diagnostic reference standard is considered standard within veterinary diagnostics (Selder et al., 2018; Waner et al., 2014). Calculating sensitivity and specificity is also considered one of the standards of evaluating the value of new medical tests. Fecal samples were sent in from reputable veterinary hospitals and animal shelters in the northern Nevada area. Due to lack of interaction with live, vertebrate animals, this study did not require IACUC approval.

Materials and Methods

Fecal Samples

Fifty dogs of varying age, sex, and breed were included in the study. All dogs presented to a local veterinary hospital or shelter, including Animal Emergency and Specialty Center, Nevada Humane Society, and Northern Nevada SPCA in Reno, Nevada and VCA Baring Boulevard Animal Hospital in Sparks, Nevada. These hospitals and shelters were chosen based on the large quantity of dogs they see regularly, as well as their responses to letters sent to 22 veterinary hospitals and shelters in northern Nevada and California. Dogs presenting with clinical signs of CPV or that tested positive for CPV with an ELISA test (n = 5) and control dogs (n = 45, no clinical signs of infection) were included. Fecal samples were collected by each clinic's kennel team and refrigerated until sent into the lab at the University of Nevada, Reno. DNA extraction and the ICPOC procedure were performed within one to three days of receiving the sample.

DNA Extraction for qPCR

The extraction of DNA from fecal samples for use in the qPCR analysis was performed using the QIAamp Fast DNA Stool Mini Kit (Qiagen), following the manufacturer's instructions for isolation of DNA from stool for detection of pathogens (Qiagen, 2014). Two-hundred milligrams of stool were measured using the spoon in each stool tube. Quantitative PCR inhibitors such as complex polysaccharides, fats, and salts were removed from the fecal samples by addition of InhibitEx buffer and homogenization of the combined sample and buffer. Lysis of bacteria and parasites was then achieved by incubation of the stool lysate at 70°C for five minutes. After centrifugation at 13,300 x g,

supernatant containing the pathogen DNA was added to proteinase K. A buffer was also added, and the solution was incubated at 70°C for 10 minutes in order to digest and degrade proteins and to optimize DNA binding to the QIAamp spin column's membrane. After the addition of ethanol, the lysate was loaded into the QIAamp spin column and DNA absorbed into the membrane by centrifugation at 13,300 x g. The DNA bound to the QIAamp membrane was then washed in two centrifugation steps using two wash buffers, again at 13,300 x g, in order to remove impurities. The purified DNA was eluted using 200 µl PCR-grade water, in place of the provided Buffer ATE, and incubated for five minutes at room temperature. This was then centrifuged at 13,300 x g for one minute to elute DNA. Extracted DNA from each sample was frozen until qPCR could be performed.

Quantitative PCR

The sequences used to design the forward and reverse primers, as well as the probe for qPCR were described in Decaro et al. (2005a). These sequences are from the VP2 nucleotide sequences consistent in known CPV-2 strains. Primers and probe were obtained from Applied Biosystems (Foster City, CA).

The 25 µl PCR mixture for one reaction contained 12.5 µl of SSOAdvanced Supermix (Biorad), 3 µl (5 µM) of forward primer (5'-AAACAGGAAT-TAACTATACTAATATATTTA-3'), 3 µl (5 µM) of reverse primer (5' -AAATTTGACCATTTGGATAAACT-3'), 0.5 µl (10 µM) of FAM probe (5'-TGGTCCTTTAACTGCATTAAATAATGTACC-3'), 2.5 µl of PCR-grade water, and 3.5 µl of extracted sample DNA. Once in the thermal cycler, DNA polymerase was activated at 95 °C for 10 minutes, then underwent 35 cycles of denaturation at 95°C for

15 seconds, primer annealing at 52°C for 30 seconds, and extension at 60°C for 30 seconds. This qPCR protocol was adapted from Decaro et al. (2005a). The entire qPCR run for all 50 samples, including two positive controls and a negative control, took one hour and 33 minutes.

In-clinic Point-of-care PCR

The ICPOC PCR test was performed according to the manufacturer's instructions. Once turned on, test type "DNA-P" was selected due to the fact that CPV is a DNA virus, and test program "M" was selected according to the reagent pouch labeling. The QubeMDx was then allowed to heat until a circular green symbol indicated that each well was ready for testing.

In initial test runs, larger fecal sample volumes resulted in invalidated internal control results. Because of this, 0.03-0.04 g of fecal material was weighed from each sample for use in the ICPOC test. Each sample applicator was then placed into the extraction tube and stirred to break up the feces sample. Following instructions, the feces sample extraction tube was shaken 10 times and allowed to stand for five minutes. When multiple samples were processed at once, extraction tubes were allowed to stand for between five and ten minutes. Each tube was then shaken 10 more times and the nucleic acid extract was dispensed into the provided Eppendorf tube by folding the extraction tube in order to squeeze the liquid into the Eppendorf. Using a micropipette, 30 µl of the nucleic acid extract from the Eppendorf tube was then put into the QubeMDx canine parvovirus detection lyophilized reagent tube, with the freeze-dried ball of reagents at the neck of the tube. The nucleic acid extract and reagents were thoroughly mixed by pipetting the mixture 30 times in order to ensure the reagents were fully dissolved.

Reagent tubes were centrifuged for one minute at 8,000 x g and checked to ensure that the fluid was clear and that the fluid level fell between the two lines on the reagent tube.

Each reagent tube was placed into the QubeMDx reaction wells until the well beeped to indicate proper placement. Once the test beeped again to indicate that the DNA amplification cycles were complete, each well indicated a result on the test screen. The "A" result corresponded to the actual CPV test conducted on the sample, while the "B" result corresponded to the internal control. When "B" was positive, the test result was validated. However, when the "B" result was negative and paired with a negative "A" result, the test was invalidated, while a positive "A" result with negative "B" result indicated a high viral concentration in the sample. Each result, once the reagent tube was placed in the well, took 15 minutes.

Most samples (n = 40) were tested using the QubeMDx twice in order to determine how reliably the QubeMDx agreed with its own result. However, this retesting did not begin until after the first set of samples (n = 10) had already been run. In addition, samples that were run twice, but had an invalidated test during one of the runs, were not rerun.

Data Analysis

Before analysis, the data gathered was prepared in an Excel sheet in order to see test runs side by side. Data was then grouped based on positive, negative, and invalidated results for each run, including the first and second tests in the QubeMDx and the qPCR results. Samples which were not tested a second time in the QubeMDx (n=11) were also noted and grouped. The data sheet was also sorted so that samples which tested the same in both QubeMDx runs could be seen clearly. This was also used to determine the

number of congruent and non-congruent positives between the QubeMDx runs and the QubeMDx and qPCR results.

Data was then analyzed using Excel. Sensitivity and specificity, as well as the positive and negative predictive values (PPV and NPV, respectively) were calculated using the number of positive and negative results from the QubeMDx and the qPCR results. Confidence intervals (CI), which serve to show the range of values the true value lies in with 95% certainty, were found using the Epitools Wilson method confidence limits calculator ("Epitools—Calculate confidence limits for a sample prop.," n.d.). Odds ratios were also calculated between the first and second QubeMDx runs and the QubeMDx and qPCR results.

Justification

Fecal samples were collected from animal hospitals and shelters in order to randomize the breeds and ages of the dogs, as well as the potential stages of disease of naturally infected animals. This was in contrast to Waner et al. (2014), in which they infected the sample dogs in-lab and were able to monitor the stages of disease each test best detected due to the amount of virus being shed. Collecting fecal samples from within the community also randomized the lifestyle conditions of each dog, which have may affected the fecal sample received by altering PCR inhibitors and other factors.

Samples were tested by both the QubeMDx and traditional qPCR to compare the positive and negative results from both machines for each sample. This method was chosen in order to directly compare the QubeMDx to the diagnostic technique which is considered the reference standard for canine parvovirus detection. This allowed the

specificity and sensitivity of the QubeMDx to be found, considering the qPCR results as the determination of a diseased versus non-diseased sample. Other in-lab tests were not compared to the QubeMDx due to the fact that qPCR is considered the best in-lab diagnostic technique for CPV (Desario et al., 2005). Other in-clinic tests were also not compared to the QubeMDx in order to contribute more clearly to the knowledge of how ICPOC qPCR compares to in-lab qPCR, rather than a different type of test such as ELISA or immunochromatography.

Limitations of the chosen methods include small sample size and some inconsistency in testing each sample in the QubeMDx twice. The small sample size was due in part to the fact that this study was conducted during the trough period for CPV, which is most prevalent during the summer months (Goddard & Leisewitz, 2010). There was also very little response from the veterinary hospitals and shelters that were contacted to obtain samples. Although over 20 hospitals and shelters received letters and calls regarding sample submission, only four responded. The other limitation was the fact that some samples did not produce two valid results in the two QubeMDx runs. However, in comparing the two runs, only those which had two validated test results were included in order to make the statistical analysis consistent.

Results

Predictive Validity

Fifteen of the fifty tested samples were found to be positive for CPV in the reference qPCR, while 35 were negative. These results were in comparison to the 21 samples which tested positive during the first QubeMDx test run, and the 16 which tested

positive out of the 40 samples that were tested during the second QubeMDx run. There were six samples that had one test result invalidated by a negative internal control, while ten samples from the first sample set were not rerun in the QubeMDx machine. This means that 34 samples had two valid test results between the first and second QubeMDx runs. Among these, there were nine congruent positives and 11 congruent negatives between both runs. This leaves 14 samples that tested positive during one of the two QubeMDx tests, but negative during the other.

Table 1. Test results from both QubeMDx runs as compared to the reference qPCR.

	Positive	Negative	Invalid Test	Total
QubeMDx Run #1	21	25	4	50
QubeMDx Run #2	16	22	2	40
qPCR	15	35	0	50

For the sake of combining the two QubeMDx test runs to compare to the qPCR results, samples which tested negative once between the two runs, as well as those which tested negative both times, were considered negative. Samples which were identified as positive both times, or which had only one valid test which was positive, were considered a positive QubeMDx result. Based on these congruent and non-congruent positives between the QubeMDx and qPCR, specificity and sensitivity were determined. There were four positives found using the QubeMDx which were negative in qPCR, indicating that the specificity of the test was 88.6% (CI 95%, 74.1%-95.5%). This means that 88.6%

of animals with negative test results are truly negative for the disease. There were also six negatives found by the QubeMDx which were found to be positive in qPCR, resulting in a sensitivity of 60.0% (CI 95%, 35.8%-80.2%). This indicates that the test was able to detect 60.0% of animals with the disease. The positive predictive value of the test was calculated to be 69.2% (CI 95%, 42.4%-87.3%), indicating that among animals that test positive for CPV in the QubeMDx, 69.2% actually have the disease. The negative predictive value was found to be 83.8% (CI 95%, 68.9%-92.4%), indicating that among animals that test negative in the ICPOC test, 83.8% do not have the disease.

Table 2. Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of the in-clinic point-of-care test (QubeMDx).

Sensitivity (CI 95%)	60.0% (35.8-80.2%)
Specificity (CI 95%)	88.6% (74.1-95.5%)
PPV (CI 95%)	69.2% (42.4-87.3%)
NPV (CI 95%)	83.8% (68.9-92.4%)

Odds ratios were calculated between the two QubeMDx runs as well as between the QubeMDx and qPCR. Based on the two QubeMDx test runs, the number of test results which were not consistent between both runs (non-congruent results) were compared to the number of samples which tested the same during both runs (congruent positives and negatives) in order to compute the odds ratios. The odds that a sample would test differently during the second QubeMDx run compared to the first QubeMDx

run were found to be 0.48. This indicates that when samples were rerun, the two QubeMDx tests would not agree nearly half of the time. Similarly, the samples on which the QubeMDx and qPCR results disagreed (non-congruent results) were compared to those in which the two tests agreed on either a positive or negative result (congruent positives and negatives). The odds that a sample would test differently in the QubeMDx than in the reference standard qPCR were found to be 0.09, indicating that the majority of samples tested the same in the QubeMDx as they did during qPCR.

Table 3. Agreement between QubeMDx Run #1 and QubeMDx Run #2 results

Congruent positives	9
Congruent negatives	11
Non-congruent results	14
Odds Ratio	0.48

Table 4. Agreement between combined QubeMDx results and qPCR

Congruent positives	9
Congruent negatives	31
Non-congruent results	10
Odds Ratio	0.09

Practicability Assessment

The practicability of the QubeMDx was good in terms of the ease-of-use and timeliness of testing each sample. DNA extraction and reagent tube preparation for each sample took between ten and fifteen minutes, with the time extended up to 18 minutes when multiple samples were being prepared at once. Once each tube was placed in one of the QubeMDx wells, a result was available within 15 minutes. The instructions for QubeMDx sample preparation and test running were very clear and simple. The pre-packaged extraction and Eppendorf tubes, as well as the reagent tube and lyophilized chemical ball, made the preparation process user-friendly, even for someone with little lab training, as might be assisting running diagnostic tests in a veterinary clinic. The screen on the QubeMDx itself also made both the test selection and results distinct and readable.

However, the ease-of-use and timeliness for running each sample were undercut by the fact that certain elements of the test were somewhat difficult, including requiring a very small amount of fecal sample and ensuring that the liquid level in the reagent tube fell within a very small range. Issues with these testing elements required frequent re-running of samples, which required starting the DNA extraction process over again. Even when instructions seemed to have been followed exactly, six of the 50 samples run resulted in an invalidated internal control for unclear reasons.

Discussion

The purpose of this study was to determine the sensitivity and specificity of an in-clinic point-of-care qPCR test (the QubeMDx) in diagnosing canine parvovirus through

comparison to the reference qPCR. This was done in order to evaluate the overall efficacy of the QubeMDx as an in-clinic diagnostic tool. Practicability of the ICPOC test was also determined by timing test runs and evaluating the test's ease of use. The results suggest that, although the test has a fairly high specificity, it suffers from low sensitivity and low repeatability when a sample is retested. The practicability of the QubeMDx was good, with quick, easy-to-perform steps and a clear user interface. However, certain more precise steps, as well as unclear negative results for the internal control, required rerunning tests, which took more time and weakened the overall practicability of the test.

The specificity of the QubeMDx was found to be 88.6% (CI 95%, 74.1%-95.5%). This is considered a fairly high specificity, although it is not in the ideal specificity range above 90%. The high specificity indicates that the rate of false positives, wherein the sample tested positive for CPV in the QubeMDx but negative in qPCR, was low. This means that samples which the ICPOC test labeled as negative were highly likely to be true negatives. Because of the high specificity of this test, positive tests in the QubeMDx would be useful for diagnosis, since the test rarely gives a positive result for a non-infected sample. However, since the specificity is not 100%, further diagnostic testing would still have to be done to confirm a positive result. This is somewhat contrary to the findings of Selder et al. (2018) and Waner et al. (2014), both of which found that the ICPOC PCR tests had 100% specificity. The few false-positive results which reduce the specificity of the QubeMDx may be due to recent vaccinations. Samples, especially those from local shelters, may have come from dogs which were vaccinated within the month before collection and were therefore continuing to shed viral DNA from the CPV vaccine in their feces (Freisl et al., 2017).

The sensitivity of the QubeMDx was found to be much lower, at 60.0% (CI 95%, 35.8%-80.2%). This low sensitivity indicates a high rate of false negatives, wherein the sample tested negative in the QubeMDx but positive during qPCR. Only 9 out of the 15 samples that were positive for CPV by the qPCR reference standard were also positive by the QubeMDx. This low sensitivity is consistent with previous findings on the specificity and sensitivity of ICPOC qPCR tests, such as in findings by Selder et al. (2018), in which the sensitivity of a given test was found to be under 60% for all tissue types, as well as in a study by Waner et al. (2014), in which the ICPOC qPCR test was found to have 30% sensitivity overall.

The low sensitivity of this test may be explained by a few possibilities. It is possible that the QubeMDx requires a relatively high concentration of viral DNA in order to generate positive results, whereas the reference qPCR was able to detect CPV in samples with a smaller viral DNA load. In a previous study by Waner et al. (2014) testing blood samples for *Ehrlichia canis* DNA, sensitivity of the ICPOC qPCR test was found to be much higher (75% compared to 30%) during the acute phase of the disease, when the bacterial DNA load is considered to be much higher. This suggests that the sensitivity of ICPOC qPCR tests may heavily depend on the concentration of pathogen DNA in the samples. It is also possible that the QubeMDx was not able to equally detect all three variants of the CPV-2 virus. However, this is not considered very likely due to a study conducted by Decaro et al. (2013), which found that common in-clinic assays were able to detect CPV-2a, -2b, and -2c at equivalent rates. Finally, it is possible that the low sensitivity of the ICPOC test could be caused by false positive results from the reference qPCR. False positives from the qPCR results could be due to the presence of CPV

vaccine shed in the feces of recently vaccinated dogs, which have been shown to shed CPV up to a month after vaccination (Freisl et al., 2017). Viral particles present in the modified live canine parvovirus vaccine might be especially likely to cause a positive qPCR result if the reference standard qPCR is able to detect CPV in samples with smaller viral loads. Initial pretests with the QubeMDx showed that the ICPOC test was able to detect vaccine DNA if present in larger concentrations than might normally be present in a vaccinated animal. In order to determine if this is the case, it would be useful to sequence the DNA from samples which were positive during qPCR in order to determine if the viral DNA present is from vaccinations or natural infection.

The odds ratios calculated based on the congruent and non-congruent results between tests serve to evaluate both the retest reliability of the QubeMDx as well as the likelihood of the QubeMDx results aligning with the reference standard qPCR test results. The odds ratio comparing the non-congruent and congruent results between the first and second QubeMDx runs considered the 34 samples for which there were two valid test results. This created odds of 0.48 of results changing between the two runs. This indicates that when samples were retested in the QubeMDx, they changed nearly half of the time. This is significant in that it suggests that the results from the ICPOC test are not consistent over time, making the test less reliable since it does not produce the same results. The odds ratio calculated based on agreement between the QubeMDx and reference qPCR results was found to be 0.09. This shows that there was a very low likelihood of a test result being different in the reference qPCR than the QubeMDx. This level of agreement between the two tests is significant in terms of an overall evaluation of the QubeMDx. However, the sensitivity and specificity values further quantify the

agreement between the two tests in order to show which kinds of test results are congruent between the reference standard and ICPOC test.

The negative predictive value (NPV) of the test, which compares the number of negatives identified by the reference standard to the number of samples the QubeMDx identified to be negative, was found to be 83.8% (CI 95%, 68.9%-92.4%). This indicates that 83.8% of the samples that the QubeMDx found to be negative were truly negative. The positive predictive value (PPV) of the test, which compares the number of positives identified by the QubeMDx to those found by reference qPCR, was found to be 69.2% (CI 95%, 42.4%-87.3%). However, accurate NPV and PPV values rely on the prevalence of canine parvovirus infection in the northern Nevada area, which is unknown.

The QubeMDx was easy to use and results were easy to read. Each test took less than 30 minutes to complete, which also presents a significant improvement over the time taken to run reference qPCR. However, the practicability of the test was limited by the number of retests required due to invalidated internal control results. Another limitation of the QubeMDx for in-clinic use may be the fact that DNA extraction and running of the test require minimization of contamination risks from other DNA. This would require in-clinic measures to ensure the environment and operation of the QubeMDx are free from other animal samples and sources of contamination to ensure reliable results.

One limitation of this study was that not all samples were able to be tested twice to generate two valid ICPOC test results so as to compare the retest reliability of the two QubeMDx runs. This was due to the fact that the first set of samples was not retested, as well as due to time constraints with invalidated tests. However, to compare the two runs, only those samples with two valid tests were included. In addition, the generalizability of

results may be limited by the small sample size. This was partially due to the fact that the trough period of canine parvovirus limited the number of animals which presented to local clinics with CPV-like symptoms.

In comparison to a study by Decaro et al. (2013) which found the sensitivity (65.3%) and specificity (100%) of a common ELISA-based ICPOC test, the QubeMDx was found to have lower specificity and sensitivity. However, no ELISA tests were performed during this study and so no direct comparison between the ICPOC qPCR test and the ICPOC ELISA test could be made. It may therefore be valuable to compare the sensitivity and specificity, as well as the retest consistency and practicability of the QubeMDx to an ELISA test in the future.

Conclusion

This research aimed to evaluate the efficacy of a new in-clinic point-of-care qPCR-based test (the QubeMDx) in detecting canine parvovirus by determining the sensitivity and specificity of the ICPOC test compared to reference qPCR, as well as by evaluating the practicability of the test. Based on analysis of test results in two QubeMDx test runs and reference qPCR, the sensitivity and specificity were found to be 60.0% (CI 95%, 35.8%-80.2%) and 88.6% (CI 95%, 74.1%-95.5%), respectively. The results indicate that, though the ICPOC qPCR test has good practicability, its ability to accurately detect canine parvovirus was lower than considered acceptable for a diagnostic test.

This thesis contributes to the growing body of work regarding the use and efficacy of new ICPOC qPCR platforms which have become more widely available over

the last five years. In-clinic testing is an important part of veterinary clinics' ability to detect and react to infectious diseases. As more ICPOC qPCR tests become available, these tests have the ability to bring molecular diagnostic tests, based on the direct detection of pathogen DNA, into veterinary clinics without the need for expensive equipment or trained personnel (Waner, 2016). However, it is important that these tests meet expectations for accuracy and ease of use. The QubeMDx could be a valuable diagnostic tool for producing results quickly when decisions about hospitalization and treatment need to be made. However, the usefulness of this platform is limited by the fact that further diagnostic steps are required to confirm the QubeMDx results.

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